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Research Paper

Role of macrophages in age-related oxidative stress and lipofuscin accumulation in mice

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ABSTRACT

The age-related changes in the immune functions (immunosenescence) may be mediated by an increase of oxidative stress and damage affecting leukocytes. Although the "oxidation-inflammation" theory of aging proposes that phagocytes are the main immune cells contributing to "oxi-inflamm-aging", this idea has not been corroborated. The aim of this work was to characterize the age-related changes in several parameters of oxidative stress and immune function, as well as in lipofuscin accumulation ("a hallmark of aging"), in both total peritoneal leukocyte population and isolated peritoneal macrophages. Adult, mature, old and long-lived mice (7, 13, 18 and 30 months of age, respectively) were used. The xanthine oxidase (XO) activity-expression, basal levels of superoxide anion and ROS, catalase activity, oxidized (GSSG) and reduced (GSH) glutathione content and lipofuscin levels, as well as both phagocytosis and digestion capacity were evaluated. The results showed an age-related increase of oxidative stress and lipofuscin accumulation in murine peritoneal leukocytes, but especially in macrophages. Macrophages from old mice showed lower antioxidant defenses (catalase activity and GSH levels), higher oxidizing compounds (XO activity/expression and superoxide, ROS and GSSG levels) and lipofuscin levels, together with an impaired macrophage functions, in comparison to adults. In contrast, long-lived mice showed in their peritoneal leukocytes, and especially in macrophages, a well-preserved redox state and maintenance of their immune functions, all which could account for their high longevity. Interestingly, macrophages showed higher XO activity and lipofuscin accumulation than lymphocytes in all the ages analyzed. Our results support that macrophages play a central role in the chronic oxidative stress associated with aging, and the fact that phagocytes are key cells contributing to immunosenescence and "oxi-inflamm-aging". Moreover, the determination of oxidative stress and immune function parameters, together with the lipofuscin quantification, in macrophages, can be used as useful markers of the rate of aging and longevity.

1. Introduction

Aging is associated with a progressive decline of physiological functions, which leads to aged-related pathologies and, ultimately, to death. The immune system is especially affected by this process, causing an increased susceptibility to infections and mortality, as well as a major incidence of immune diseases and cancer in the elderly [1–3]. The age-related impairment of the immune system, which is referred to as immunosenescence, involves remodeling changes in its organization and functionality that negatively impact the health of older adults [1,2,4]. Although the immune cells change their functional competence with aging, not all immune cell types or all functions of an immune cell show a significant impairment. In fact, a decrease in several lymphocyte functions (e.g. chemotaxis and proliferation) has been described, but an increase in other functions, especially those carried out by phagocytic cells (e.g. digestion capacity, ROS production), has also been observed [1–4]. In addition, it has been proposed that the basis of immunosenescence, is the same as that responsible for the senescence of other cells in the organism, namely the chronic oxidative stress, linked to the unavoidable use of oxygen to support cellular functions, and the consequent damage [2–4].

The immune cells continuously generate oxidants and inflammatory compounds, in order to carry out their defensive functions. The free radicals and the reactive oxygen or nitrogen species (ROS and RNS, respectively), which are produced by mitochondria, but especially in different metabolic processes, are necessary for the destruction of

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pathogens and tumor cells [1,2,4]. This fact occurs principally in phagocytes (e.g. macrophages, neutrophils, etc.), in which high amounts of ROS (mainly superoxide anion and hydrogen peroxide) are produced in order to carry out the microorganism destruction. This process involved the "respiratory burst" with participation of enzymes such as NADPH oxidase and xanthine oxidase (XO) [5,6]. However, although the ROS and RNS are beneficial to maintain both cellular signal transductions and functions, and therefore are necessary to maintain homeostasis, when they are up-regulated, their higher concentrations may contribute to increase the oxidative stress and the consequent damage in cells and tissues [2–4]. To avoid these harmful effects, in the immune cells and in the surrounding cells, leukocytes generally have higher concentrations of antioxidant enzymes (e.g. catalase, etc.) and non-enzymatic compounds (e.g. glutathione, etc.) than other cells [7,8]. Thus, in the immune cells more than in other cells, a perfect oxidant-antioxidant balance is necessary [4,9]. Furthermore, it is important to note that immune cells also produce inflammatory compounds for their defensive functions, however, if it is production is not well-controlled by anti-inflammatory compounds, this leads to an inflammatory stress situation. Because of both oxidation and inflammation are interlinked process and have many feedback loops [10], an excessive production of ROS and RNS by leukocytes could also induce an inflammatory response in these immune cells [11]. Thus, ROS can also activate the transcription factor NF-KB, which stimulates the expression of genes programming the production of inflammatory mediators in leukocytes (e.g. proinflammatory cytokines, chemokines, etc.) and these could also induce an oxidative stress situation [10,12]. If these pathways are not well regulated, a vicious circle of oxidation-inflammation could be established, which would increase the oxidative stress associated with aging, and consequently accelerate this process [3,4,10]. In this context, the "oxidative-inflammatory" theory of aging was proposed [2]. This theory suggested that the age-related oxidative-inflammatory stress affects all cells, but especially those of the regulatory systems, such as the nervous, endocrine and immune systems, resulting in an impairment of their functions, as well as in the communication between them. Moreover, this theory proposes a key involvement of the immune system in "oxi-inflamm-aging", and therefore in the rate of aging in the organism [2,3,12]. Thus, the age-related impairment of immune functions may be mediated by an increase of both oxidative stress and cellular accumulation damage affecting leukocytes. In this regard, previous studies of our group revealed that the higher oxidative stress state (e.g. increased intracellular superoxide anion, oxidized glutathione, XO activity, etc.) and oxidative damage (e.g. increased levels of malondialdehyde, 8-oxodeoxyguanosine, etc.) observed in leukocytes from old mice, as well as in peripheral blood immune cells from older humans, were coincident with the impaired immune responses (e.g. phagocytosis, chemotaxis, lymphoproliferation, etc.), whereas healthy human centenarians and long-lived mice, who show preserved immune functions, show a lower expression of proinflammatory genes and a well-controlled oxidative stress in their immune cells, which could explain their successful aging [2,12–14]. In this context, it is important to note that phagocytes, such as macrophages and neutrophils, have been proposed to be the main cells responsible for the chronic oxidative-inflammatory stress associated with immunosenescence [1,2]. Thus, as a result of the age-related oxidative injury, these cells could lose their capacity to regulate their own redox and inflammatory balance, producing more and more oxidant and inflammatory compounds, and therefore, contributing to the increased oxidative-inflammatory stress observed in other physiological systems with aging. However, although some studies conducted on peritoneal macrophages and peripheral blood neutrophils, from mice and humans, respectively, have showed that these cells generate higher levels of oxidant compounds than those produced by lymphocytes, and these levels significantly increase with age [1,2], this idea has been scarcely studied.

Oxidative stress leads to an elevated oxidation of macromolecules, such as DNA, lipids, and proteins. It was postulated that age-related accumulation of damaged, oxidized, and aggregated compounds might contribute to the aging process. In this regard, lipofuscin is considered one of the best "hallmark of aging", due to the fact that the amount of lipofuscin increases with age, but also, and more importantly because the rate of lipofuscin accumulation correlates negatively with longevity [15–17]. Lipofuscin and lipofuscin-like compounds, are formed by polymeric substances, primarily composed of cross-linked lipid and protein residues due to iron-catalyzed oxidative processes, and should be regarded as aggregates of undigested cell materials [17]. The ineluctable formation and accumulation of lipofuscin in, especially, postmitotic cells, seems to lead to a variety of defects in cellular function and homeostasis. The indigestible nature of this material is associated with progressive diminution of lysosomal function and alterations in both phagocytosis and autophagy processes, which has secondary effects on many different cellular activities [17]. Moreover, it has been described that higher accumulation of lipofuscin may promote generation of ROS, sensitizing cells to oxidative injury through lysosomal destabilization, making cells considerably more vulnerable to oxidative stress [17]. Although it has been observed that lymphocytes from aged mice showed abundant granules of lipofuscin [1] and it is known that lipofuscin may act as danger signals, stimulating the release of proinflammatory chemokines and cytokines, contributing to the activation of macrophages, and therefore, leading to chronic oxidative-inflammatory process [18], to our knowledge, there are no studies about the age-related variations of the lipofuscin accumulation in phagocytes, and even less in comparison to other immune cell types.

In view of all of the above, it seems evident that immune cells, and especially macrophages, can play an important role in the increase oxidative-inflammatory stress and damage associated with aging, which could also contribute to the impairment of the immune functions, and the increase of the rate of aging. Therefore, the aim of this study was to investigate the age-related changes in several parameters of oxidative stress and immune functions, as well as in the lipofuscin accumulation, in isolated peritoneal macrophages of mice, as well as in the total population of peritoneal leukocytes, containing mainly B and T lymphocytes, macrophages, and NK cells. For this purpose, the total population of peritoneal leukocytes, as well as both isolated macrophages and lymphocytes from ICR/CD1 female mice of different ages, that is, adult, mature, old and natural extreme long lived were used, in order to analyze a variety of oxidant compounds (XO activity and expression, intracellular superoxide anion and ROS levels and oxidized glutathione content) and antioxidant protectors (namely catalase activity and reduced glutathione levels), as well as their redox balance (XO/CAT and GSSG/GSH ratios). Furthermore, the degree of lipofuscin accumulation in both isolated macrophages and lymphocytes was measured.

2. Materials and methods

2.1. Animals and experimental conditions

For this study, ex-reproductive females of outbred ICR/CD-1 mice (*Mus musculus*) were used, purchased from Harlan Interfauna Ibérica (Barcelona, Spain) at the young adult age (28 ± 4 weeks of age). The mice were specifically pathogen free as tested by Harlan according to the Federation of European Laboratory Science Association recommendations. They were housed 6 ± 1 per cage, at a constant temperature (22 ± 2 °C), in sterile conditions inside an aseptic air negative-pressure environmental cabinet (Flufrance, Cachan, France) and on a 12/12 h reversed light/dark cycle. All animals had access to tap water and standard Sander Mus pellets (A04 diet from Panlab L.S. Barcelona, Spain) *ad libitum*. This diet was in accordance with the recommendations of the American Institute of Nutrition for laboratory animals. This cross-sectional study was performed simultaneously on mice of different ages, namely adult (28 ± 4 weeks, n=12), mature (52 ± 4 weeks, n=12), old (72 ± 4 weeks, n=10) and long lived (122 ± 2 weeks, n=7). The long-lived mice had naturally achieved healthy and successful aging because the average life span for females of ICR/CD1 mice strain is 91.9 ± 5.6 weeks [12]. Before any experimental procedures, mice were acclimated for 7 days. The protocol was approved by the Experimental Animal Committee of the Complutense University of Madrid (Spain). Animals were treated according to the guidelines of the European Community Council Directives 2010/63/EU.

2.2. Collection of peritoneal leukocytes

Peritoneal suspensions were collected between 8–10 a.m., to minimize circadian variations, without killing the animals. Mice were held by the cervical skin, the abdomen was cleansed with 70% ethanol and 3 ml of sterile Hank's solution, previously tempered at 37 °C, was injected intraperitoneally. After massaging the abdomen, 80% of the injected volume was recovered. Peritoneal leukocytes, mainly lymphocytes, macrophages and NK cells were identified by their morphology and quantified in Neubauer chambers using optical microscopy (40x). Cellular viability, routinely checked before and after each experiment by the trypan blue (Sigma-Aldrich, Spain) exclusion test, was higher than 99 ± 1% in all cases. The peritoneal suspensions were adjusted to a specific number of total leukocytes, depending on the parameter analyzed as described in the corresponding section. Additionally, aliquots of the cellular suspensions were used to confirm the leukocytes counts by flow cytometry.

2.3. Peritoneal leukocytes population analysis

Percentages of CD11b (leukocyte differentiation antigen typical of macrophages), CD19 (B lymphocytes) and CD3 (T lymphocytes) cells present in the sample were analyzed by flow cytometry. Monoclonal antibodies (BD Biosciences, CA, USA) were used for superficial staining at the following final concentrations in the tube: peridinchlorophyll(PerCP)-labeled anti-CD45 (0.05 μg/10^6 cells); fluorescein isothiocyanate (FITC)-labeled anti-CD11b (1.25 μg/10^6 cells), anti-CD19 (1 μg/10^6 cells); allophycocyanin (APC)-labeled anti-CD3 (0.25 μg/10^6 cells). The peritoneal leukocytes were washed at 400*g* for 10 min and adjusted to 2×10^5 leukocytes/ml in phosphate buffer saline (PBS, pH 7.4, Sigma-Aldrich) with 1% bovine serum albumin (BSA, Sigma-Aldrich), which was used to avoid non-specific binding. Cells were then centrifuged at 5800*g* for 10 min, supernatants were discarded and antibody mixtures (30 μl) were added to each tube. PBS-BSA was added to blank tubes. Cells were incubated with the antibodies for 30–45 min at 4 °C in dark, and later were washed twice at 580*g* for 5 min in PBS-BSA to remove free antibodies. Cells were acquired on the flow cytometer (FACScalibur Flow Cytometer, Becton-Dickinson, Franklin Lakes, NJ, USA). Results were analyzed with Cell Quest Pro software (BD Biosciences) and expressed as percentage (%) of CD11b, CD19 and CD3 cells with regard to the number of CD45+ cells present in the sample.

2.4. Isolation of peritoneal macrophages

To isolate peritoneal macrophages, the peritoneal suspensions were adjusted to 3×10^6 cells/ml in RPMI 1460 without phenol red, and were added to 24-well culture plates. Cells were incubated at 37 °C for 2 h to allow adherence of macrophages to the plastic surface. After this time, the supernatants containing non-adherent cells (mainly lymphocytes) were removed by gently washing three times with warm PBS 1X and were centrifuged at 400*g* for 10 min and were resuspended in Hank's solution for counting and viability. Furthermore, adherent cells (macrophages) were detached from the plastic by trypsinization process. Cells were incubated at 37 °C for 30 min with a solution of trypsin 0.25% containing ethylene diamine tetraacetic acid (EDTA) 1 mM (1 ml). Afterwards, PBS 1X containing fetal bovine serum (20% FBS, Gibco) was added to inactivate trypsin. Adhered macrophages were gently scraped with a cellular scraper to remove them from the plastic. The collected cell suspensions were washed at 400*g* for 10 min at 4 °C and were resuspended in Hank's solution for counting and viability. Finally, the cells were characterized by flow cytometry using the procedure described in Section 2.3. It was found that 90–95% of the adherent cells were macrophages. The macrophages suspensions were adjusted to a specific number of cells, depending on the parameter analyzed as described in the corresponding section.

2.5. Xanthine oxidase activity assay

Xanthine oxidase (XO) activity was assayed in total peritoneal leukocytes, as well as in isolated peritoneal macrophages and non-adherent cells (mainly lymphocytes) using a commercial kit (A-22182 Amplex Red Xanthine/Xanthine Oxidase Assay Kit, Molecular Probes, Paisley, UK). In the assay, XO catalyzes the oxidation of purine bases (xanthine/hypoxanthine) to uric acid and superoxide anion. The superoxide spontaneously degrades in the reaction mixture to hydrogen peroxide, which in the presence of horseradish peroxidase (HRP), reacts stoichiometrically with Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin. Aliquots of total leukocytes, macrophages or lymphocytes adjusted to 10^6/ml were lysated in potassium phosphate buffer (0.05 M, pH 7.4) containing EDTA (0.1 M, pH 7.4) and dithiothreitol (DTT 0.5 mM, pH 7.4; to prevent reversible XDH to XO conversion), and finally normalized according to total protein. In the assay, 50 μl of the lysated were incubated with 50 μl working solution of Amplex Red reagent (100 μM) containing HRP (0.4 U/ml) and xanthine (200 μM). After 30 min of incubation at 37 °C, measurement of fluorescence was performed in a microplate reader (Fluostar Optima, BMG Labtech, Biomedal, Spain) using excitation and emission detection at 530 and 595 nm, respectively. The XO (10 mU/ml) supplied in the kit was used as the standard, and XO activity was measured by comparing the fluorescence of samples with that of standards. Protein content of the same samples was evaluated following bicinchoninic acid (BCA) protein assay kit protocol (Sigma-Aldrich, Spain). The results were expressed as international milliunits (mU) of enzymatic activity per milligram of protein (mU XO/mg protein).

2.6. Analysis of xanthine oxidase expression

Proteins were extracted from total peritoneal leukocytes and isolated peritoneal macrophages (adjusted to 3×10^6 cells/ml) in ice-cold lysis buffer (10 mM Tris-HCl buffer, 150 mM NaCl, 1 M NaF, 100 μM sodium orthovanadate, 10 mM ethylenediamine tetraacetic acid, 1% triton X-100, 250 mM PMSF, 10 mM DTT, and 5 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin), incubated for 10 min on ice, and centrifuged at 5000*g* for 20 min at 4 °C. The protein concentration of the supernatant was determined (BCA assay kit; Sigma-Aldrich) and 50 μg of protein was loaded for 8% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes, blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20% and 5% dry milk, overnight incubated with the polyclonal anti-XO antibody (150 kDa, 1:2000; Abcam, UK). The membranes were washed 3 times (10 min each) with TBS containing 0.1% Tween-20 for 10 min. The same membranes were used to determine β-actin expression as loading control, using a polyclonal antibody anti-β-actin (45 kDa, 1:4000; Abcam, UK). For detection, HRP peroxidase conjugated secondary antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. Protein bands were visualized with the ECL Plus Western blotting Luminol Reagent (Santa Cruz Biotechnology, USA). Proteins were detected using an enhanced chemiluminescence western blotting analysis system (Pierce, Rodkford, IL, USA). XO protein bands were analyzed by densitometric analyses and normalized against the intensity of β-actin. The results were expressed as densitometry (DO) arbitrary units.

2.7. Intracellular superoxide anion measurement

The intracellular superoxide anion ($O_2^{\bullet-}$) levels were assessed by nitro blue tetrazolium (NBT) assay, which detects reduction of NBT to formazan by $O_2^{\bullet-}$ anion. The assay was carried out following the method previously described [19] with a slight modification [20]. Briefly, aliquots (200 µl) of total peritoneal leukocytes or isolated macrophages adjusted to 10^6 cells/ml, were mixed with NBT (1 mg/ml) and with Hank's solution (basal conditions) or with phorbol 12-myristate 13-acetate (PMA; 5 ng/ml; Sigma-Aldrich) diluted to 1% in Hank´s solution (stimulated conditions). After, 60 min of incubation at 37 °C, the reaction was stopped with HCl 0.5 M (Sigma-Aldrich) and the samples were centrifuged 30 min at 1600*g*. The supernatants were discarded and the intracellular reduced NBT extracted with dioxan (Sigma-Aldrich). After centrifugation, the absorbance of the supernatants was determined at 525 nm in a spectrophotometer. The results of the levels of $O_2^{\bullet-}$ anion were expressed as nmol $O_2^{\bullet-}/10^6$ cells by extrapolating from a standard curve of NBT reduced with 1,4-dithioerythriol (Sigma-Aldrich). Moreover, the percentage of stimulation in response to PMA was calculated, with non-stimulated values being 100%.

2.8. Intracellular ROS measurement

The level of intracellular ROS was assessed by fluorescence microplate reader following the probe of the 2′, 7′-dichlorofluorescein diacetate (DCFH-DA), which oxidizes to fluorescent dichlorofluorescein (DCF) in the presence of ROS, as described previously [20]. Briefly, aliquots (200 µl) of total peritoneal leukocytes or isolated peritoneal macrophages adjusted to 10^6 cells/ml in Hank's medium were loaded into a 96-well plate in triplicate and incubated for 30 min (37 °C) with DCF-DA (1 mM; Sigma-Aldrich) in darkness. Afterwards, cells were incubated 30 min (37 °C) with PMA (5 ng/ml), which were used as positive controls for stimulation of ROS production, while spontaneous ROS levels was measured in the presence of Hank's medium (basal conditions). Fluorescence of the samples was recorded with excitation and emission wavelengths at 485 nm and 535 nm, respectively. The resulting data were normalized using the control values and the results were expressed as the stimulation index, which is the percentage of stimulation in response to PMA, with non-stimulated values being 100%.

2.9. Catalase activity assay

Catalase activity was determined following the method described by Beers and Sizer [21], with the following modifications, and based on the decrease of the absorbance at 240 nm because of the decomposition of hydrogen peroxide (H_2O_2) by the enzyme. The total peritoneal leukocytes or peritoneal macrophages suspensions were previously adjusted to 10^6 cells/ml in Hank's medium and aliquots of 1 ml were used to perform the enzymatic assay. The cells were centrifuged at 1100*g* for 10 min at 4 °C, and the pellets were resuspended in 50 mM phosphate buffer, containing H_2O_2 14 mM (Merk, Germany). Then, the samples were sonicated and centrifuged at 3200*g* for 20 min at 4 °C. The enzymatic assay was followed spectrophotometrically at 240 nm through the decomposition of H_2O_2 into H_2O+O_2. Protein content of the same samples was evaluated following BCA protein assay kit protocol (Sigma-Aldrich). The results were expressed as units (U) of enzymatic activity per milligram of protein (U/mg protein).

2.10. Glutathione content assay

Both reduced (GSH) and oxidized (GSSG) forms of glutathione were assayed by the enzymatic recycling method of Tietze [22], with the following modification, by monitoring the change in absorbance at 412 nm. Briefly, aliquots of both total peritoneal leukocytes and isolated macrophages adjusted to 10^6 cells/ml in Hank´s solution were centrifuged at 1200*g* for 10 min at 4 °C. Pelleted cells were resuspended in previously degassed media, containing 5% of trichloroacetic acid in HCl 0.01 N for GSH measurements, and phosphate buffer 50 mM pH 7.0 plus EDTA (1 mM; Sigma-Aldrich) and N-ethylmaleimide (NEM, 12.5 mM; Sigma-Aldrich) to block reduced GSH for samples aimed to measure GSSG. Then, samples were sonicated and centrifuged at 3200*g* for 5 min at 4 °C. Aliquots of supernatants of immune cells were measured using the reaction mixture: 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, 6 mM; Sigma-Aldrich), β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH, 0.3 mM; Sigma-Aldrich) and glutathione reductase (10 U/ml; Sigma-Aldrich). The reaction was monitored for 240 or 140 s for GSH or GSSG determinations, respectively. Protein content of the samples was determined following BCA protein assay kit protocol (Sigma-Aldrich). The results were expressed as nmol/mg protein. Moreover, the coefficient GSSG/GSH was calculated in each sample.

2.11. Lipofuscin quantification

Lipofuscin accumulation was determined in both isolated peritoneal macrophages and lymphocytes. For that, aliquots of 200 µl of peritoneal leukocytes suspensions adjusted to 1×10^6 cells/ml were incubated on migration inhibition factor (MIF) plates (Kartell, Noviglio, Italy) for 30 min at 37 °C in a humidified atmosphere. After this time, the supernatant containing non-adherent cells (mainly T lymphocytes) were removed by gently washing two times with warm Hank´s solution. The adherent monolayer cells (mainly macrophages) were detached from the plastic by a cell scraper and then removed by gently washing two times with warm Hank's solution. The collected cell suspensions were washed at 400*g* for 10 min at 4 °C and were resuspended in Hank's solution (500 µl). Afterwards, lymphocytes and macrophages samples were fixed in a formaldehyde solution (4% in PBS; Sigma-Aldrich). Finally, cell suspensions (50 µl) were dropped onto poly L-lysine coated slides (Sigma-Aldrich), air-dryed for 5 min and washed in PBS. Slides were mounted in Vectashield antifading mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and examined with an Olympus BX61 microscope. In situ lipofuscin autofluorescence was detected with excitation and emission wavelengths of 490 nm and 515 nm, respectively. All Images were captured at the same exposure time (1000 ms) with an Olympus DP70 digital camera using the analysis software (Soft Imaging System, Olympus). Lipofuscin load was analyzed in 20 lymphocytes and 10 macrophages from each mouse, and 5 individuals from each group of age were used, except for the long-lived ones where only 3 individuals were recovered. Cells within the same individual were randomly chosen from five different fields in the slide. Measurements of lipofuscin load were made with the open source ImageJ (National Institute of Health, http://rsb.info.nih.gov/ij). The total intensity of autofluorescence per image (expressed in URF), the mean of the number of lipofuscin granules, the total area of granules (pixel^2) and the relative cell area occupied by lipofuscin granules (%) were determined. First, each autofluorescence image was converted to a 8-bit image and a region of interest (ROI) was outlined in the 2D images following the contour of the cell. For measuring the intensity of fluorescence, each pixel in the 8-bit gray-scale images was given a number between 0 and 255, corresponding to the 256 gray levels, 0 being absence of fluorescence. To analyze the rest of the parameters, digitalized images were binarised and the threshold pixel intensity range adjusted to capture the fluorescent granules. Data were collected and exported to Microsoft Excel for further analysis.

2.12. Phagocytosis assay

The phagocytosis assay was carried out following the method previously described for ingestion of inert particles (latex beads, 1.1 µm means particle size, Sigma-Aldrich) [19], with slight modifications. Aliquots of 200 µl of total peritoneal leukocytes adjusted to 1×10^6 cells/ml were incubated on MIF plates (Kartell) for 30 min at 37 °C in a humidified atmosphere. Later, 20 µl of latex beads (1.1 µm diluted to 1% PBS, Sigma-Aldrich) were added. After 30 min of incubation under the same conditions, the plates were washed, fixed with methanol (50%) and stained with Giemsa's solution (Sigma-Aldrich). The number of particles ingested by 100 macrophages was determined by optical microscopy (100x) as phagocytosis index.

2.13. Protein concentration

Protein concentration of the samples was measured following BCA assay kit protocol (Sigma-Aldrich).

2.14. Statistical analysis

Analyses were performed with SPSS 21.0 (SPSS, Chicago, IL, USA) software. All data are presented as means ± standard deviation (SD). Normality of the samples and homogeneity of the variances were checked by the Kolmogorov-Smirnov test and Levene test, respectively. Differences due to age were studied through the Student *t*-test or one-way analysis of variance (ANOVA) followed by post hoc tests analysis. The Tukey test was used for post hoc comparisons when variances were homogeneous, whereas its counterpart analysis Games-Howell was used with unequal variances when they were not homogeneous. For the lipofuscin quantification the statistical analyses were performed using the Statgraphics Centurion XVII™ program. Differences were analyzed using a one-way ANOVA or the non- parametric Kruskal-Wallis test. The LSD multiple comparisons test was applied as a post hoc test. A value of $P < 0.05$ was considered statistically significant in all cases.

3. Results

3.1. Age-related changes in xanthine oxidase (XO) activity and expression

Since XO is involved in both $O_2^{\bullet-}$ and H_2O_2 production, to confirm its role in aging, both XO activity and expression levels were evaluated in the total population of peritoneal leukocytes as well as in macrophages and lymphocytes isolated from adult, mature, old and long-lived mice. The results showed an age-related increase in the XO activity in the total population of leukocytes (Fig. 1A.1), as well as in macrophages (Fig. 1B.1), however no differences were observed in the lymphocyte population. Thus, it is important to note that old mice showed a significant increase in XO activity in total peritoneal leukocytes and macrophages that those observed in adult ($P < 0.001$ and $P < 0.01$, respectively) and mature ($P < 0.01$ and $P < 0.05$, respectively) mice, in which also a significant elevation of this parameter was observed with respect to adult mice ($P < 0.05$). In contrast, the naturally long-lived mice showed lower XO activity in both peritoneal cell populations than the old animals ($P < 0.01$ and $P < 0.001$, respectively), being similar to those found in the adult mice (Fig. 1A.1 and 1B.1). Interestingly, in isolated peritoneal lymphocytes, XO activity did not vary at the different ages studied, similar values being obtained between adult (0.73 ± 0.21 mU XO/mg protein), mature (0.75 ± 0.12 mU XO/mg protein), old (0.61 ± 0.09 mU XO/mg protein) and long-lived (0.69 ± 0.17 mU XO/mg protein) mice.

The XO protein expression was also quantified in both total peritoneal leukocytes and isolated macrophages. After the Western blot assay, a single band of approximately 150 kDa was visible for the two immune cell populations analyzed (Fig. 1A.2 and 1B.2). The quantitative densitometric analysis showed that the age-related XO expression variations in total leukocytes and macrophages follow the same pattern as that found in XO activity. Thus, higher levels of XO expression were detected in both total leukocytes and macrophages of old mice ($P < 0.01$) compared to those of adult animals, whereas in mature mice this significant increase of XO expression ($P < 0.05$) was only observed in total leukocytes. Long-lived mice showed in the total peritoneal leukocytes and macrophages lower levels of XO expression than old animals ($P < 0.05$), and similar to those in adults (Fig. 1A.2 and 1B.2). The expression of XO could not be assessed in the lymphocyte population because the number of cells obtained was not sufficient to perform the assay.

3.2. Age-related changes in intracellular oxidized compounds

Both XO and NADPH oxidase are the main enzymes involved in $O_2^{\bullet-}$ and H_2O_2 production in the oxidative burst of phagocytes. Due to the age-related increase in XO activity and expression observed in macrophages of old animals, and their preservation in long-lived mice, the intracellular levels of these oxidant species was also analyzed in both total peritoneal leukocytes and macrophages from adult, mature, old and long-lived mice (Fig. 2 and Fig. 3). The $O_2^{\bullet-}$ levels were measured in basal conditions, whereas ROS production was quantified under stimulated conditions, using phorbol myristate acetate (PMA), which is a powerful activator of macrophages.

The basal $O_2^{\bullet-}$ levels in the total population of peritoneal leukocytes (Fig. 2A), as well as in isolated macrophages (Fig. 2B), from mature ($P < 0.05$) and old ($P < 0.01$ and $P < 0.001$, respectively) mice was significantly increased compared with adults, and also with mature mice ($P < 0.05$) in the case of macrophages. Interestingly, it is important to note that macrophages from old mice showed higher levels of this radical in relation to those observed in the unfractionated peritoneal leukocyte isolates containing mainly lymphocytes, macrophages, and NK cells. Similar results were observed in PMA-stimulated ROS production (mainly intracellular H_2O_2 production, because DCF-DA fluorescence was used for detection) (Fig. 3). Thus, old mice also showed higher levels of ROS in their total peritoneal (Fig. 3A) and macrophages (Fig. 3B) than those observed in adult ($P < 0.01$) and mature ($P < 0.05$ and $P < 0.01$, respectively) mice. However, the percentage of ROS production observed in old animals was more marked in the presence of all leukocyte peritoneal populations than in isolated macrophages. Finally, total peritoneal leukocytes and macrophages from extreme long-lived mice showed levels of $O_2^{\bullet-}$ and ROS similar to those observed in adults, and significantly lower than those found in old ($P < 0.05$ and $P < 0.001$, respectively) and mature ($P < 0.05$ only in macrophages) animals.

The glutathione cycle plays an important role in the maintenance of the intracellular redox state. More than 90% of the glutathione contained in cells is in the reduced form GSH, being the principal intracellular non-enzymatic antioxidant, whereas the oxidized form GSSG represents an important oxidant compound [23]. Therefore, the age-related changes in intracellular content of GSSG, GSH as well as the GSSG/GSH ratios were also assessed in both total peritoneal leukocytes and macrophages from adult, mature, old and long-lived mice. In this section, only the results corresponding to the GSSG content will be displayed (Fig. 4). Thus, the levels of GSSG in total peritoneal leukocytes (Fig. 4A) from mature and old animals was significantly increased ($P < 0.01$ and $P < 0.05$, respectively) compared with adults, but in isolated macrophages (Fig. 4B). In macrophages, comparing to adult mice, a significant increase in GSSG content were observed in mature ($P < 0.05$) and old ($P < 0.01$) mice, in which also a significant elevation of this parameter was observed with respect to mature mice ($P < 0.05$). In contrast, extreme long-lived mice showed in both cells peritoneal populations similar GSSG content that adults, and significantly lower than those observed in old ($P < 0.01$ and $P < 0.05$, respectively) and mature ($P < 0.01$ only in total leukocytes) mice.

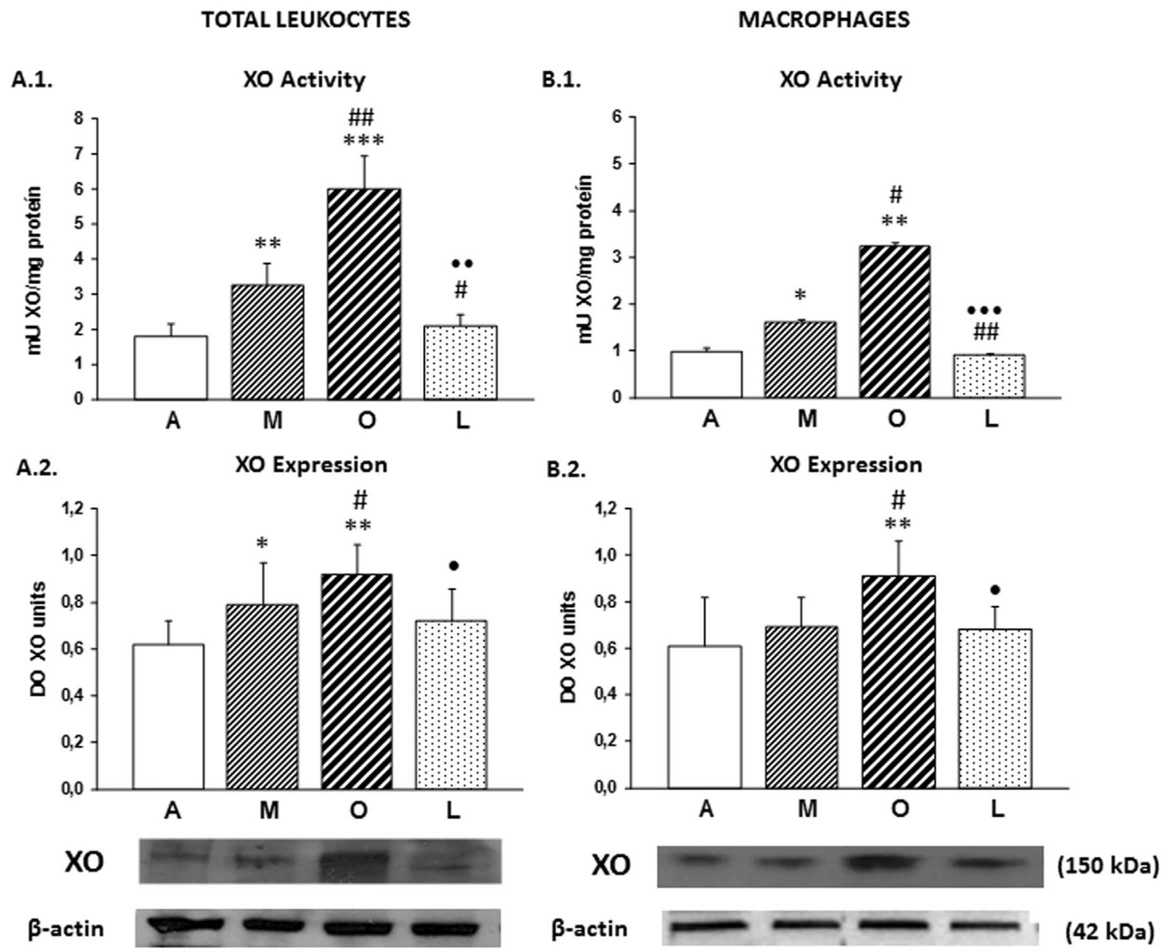

Fig. 1. Xanthine oxidase activity (1) (mU XO/mg protein) and expression (2) (XO DO/mg protein) in both total peritoneal leukocytes (A) and isolated peritoneal macrophages (B) from adult, mature, old and long-lived ICR-CD1 female mice. Data represent the mean ± SD of 6–10 values and each value being the mean of duplicate assays. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ with respect to the value in adult mice. # $P < 0.05$ and ## $P < 0.01$ with respect to the value in mature mice. •$P < 0.05$, ••$P < 0.01$ and ••• $P < 0.001$ with respect to the value old mice.

3.3. Age-related changes in antioxidant defenses

Antioxidant enzymes are essential for the preservation of the redox balance in immune cells, and in a relevant manner in phagocytes, which are constantly exposed to the high amounts of ROS that they produce during oxidative burst. Therefore, the age-related changes in the intracellular catalase activity from total peritoneal leukocytes and isolated macrophages were evaluated (Fig. 5). Catalase detoxifies of H_2O_2, protects against severe oxidative stress and is determinant in longevity. Thus, an increased catalase activity in immune cells has specifically been related to longevity in both mice and humans [24]. Old mice showed lower catalase activity in total peritoneal leukocytes and macrophages ($P < 0.01$) than the adults. In contrast, long-lived mice showed in their peritoneal leukocytes (Fig. 5A) and macrophages

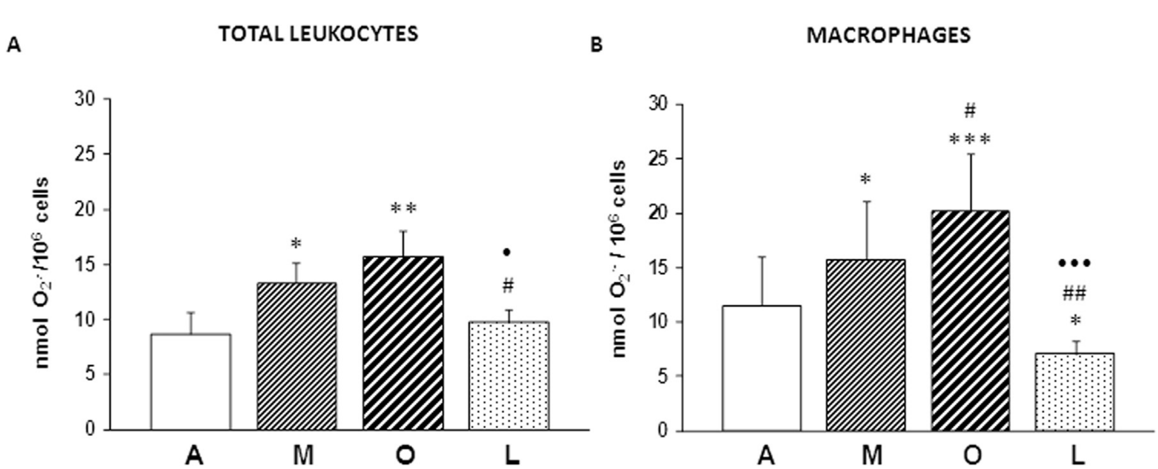

Fig. 2. Basal intracellular superoxide anion production (nmol/10^6 cells) in both total peritoneal leukocytes (A) and isolated peritoneal macrophages (B) from adult, mature, old and long-lived ICR-CD1 female mice. Data represent the mean ± SD of 6–10 values and each value being the mean of duplicate assays. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ with respect to the value in adult mice. # $P < 0.05$ and ## $P < 0.01$ with respect to the value in mature mice. •$P < 0.05$ and ••• $P < 0.001$ with respect to the value in old mice.

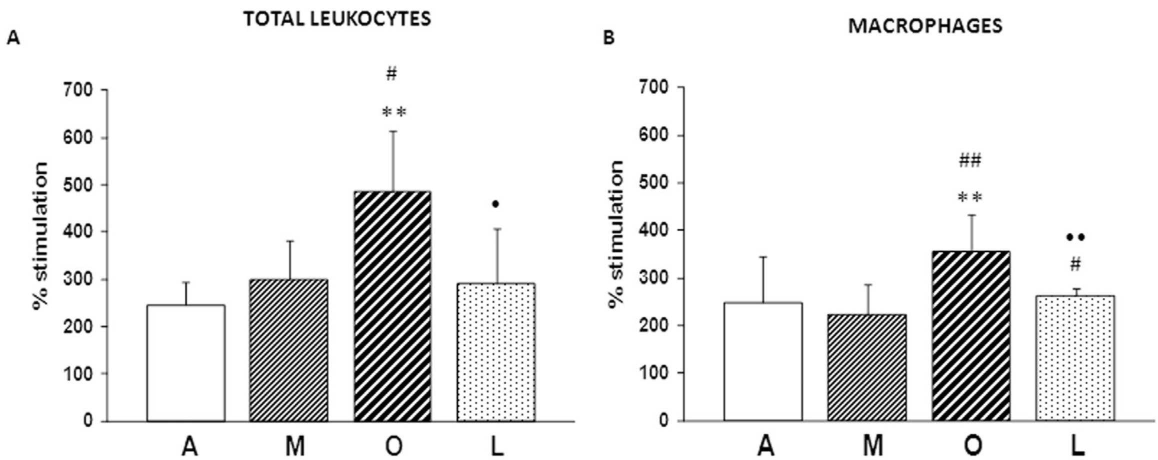

Fig. 3. Intracellular ROS stimulated production (% URF in response to PMA) in both total peritoneal leukocytes (A) and isolated peritoneal macrophages (B) from adult, mature, old and long-lived ICR-CD1 female mice. Data represent the mean ± SD of 6–10 values and each value being the mean of duplicate assays. **$P < 0.01$ with respect to the value in adult mice. # $P < 0.05$ and ## $P < 0.01$ with respect to the value in mature mice. •$P < 0.05$ and •• $P < 0.01$ with respect to the value in old mice.

(Fig. 5B) a significant increase in the catalase activity ($P < 0.01$) as compared to old animals, and even significantly higher than the adult ($P < 0.05$). However, this significant increase of catalase activity was statistically significant in total population of leukocytes but not in macrophages.

As mentioned above, the GSH is one the principal intracellular non-enzymatic antioxidant and plays a major role in maintenance of the intracellular redox state [23]. The levels of GSH in total peritoneal leukocytes from old mice (4.67 ± 1.07 nmol/mg protein) was significant decreased compared with adult (6.37 ± 1.25 nmol/mg protein; $P < 0.05$) and mature (7.05 ± 1.12 nmol/mg protein; $P < 0.01$) mice. However, the GSH content of macrophages was significantly decreased in both mature (2.63 ± 0.93 nmol/mg protein; $P < 0.05$) and old (1.73 ± 0.79 nmol/mg protein; $P < 0.01$) mice as compared to the values observed in adults (4.48 ± 0.93 nmol/mg protein). Interestingly, GSH levels was found to be decreased in total leukocytes from long-lived mice (4.95 ± 0.47 nmol/mg protein) in comparison with adult and mature ($P < 0.05$) animals, but not in their macrophages (5.08 ± 1.36 nmol/mg protein), in which they showed values similar to adults, and significantly higher than those observed in the macrophages of mature and old mice ($P < 0.05$ and $P < 0.01$, respectively).

3.4. Redox balance in total peritoneal leukocytes and isolated macrophages

As mentioned previously, the GSSG/GSH imbalance is one of the best markers of oxidative stress. In general, an age-related GSSG/GSH increased progression was observed in peritoneal leukocytes (Fig. 6). Thus, in total peritoneal leukocytes (Fig. 6A.1) and macrophages (Fig. 6B.1), the GSSG/GSH ratios observed in old mice were significant higher than those in adult ($P < 0.01$ and $P < 0.001$, respectively) and mature ($P < 0.05$) mice. Interestingly, long-lived mice showed in their total leukocytes and macrophages a GSSG/GSH ratio significantly lower than old ($P < 0.01$ and $P < 0.001$, respectively) and mature ($P < 0.05$ only in macrophages) mice, and being similar to the adult (Fig. 6A.1 and 6B.1).

Furthermore, even that the H_2O_2 is the main ROS produced by XO [25], the imbalance between XO and CAT may be an important indicator of the redox state associated with aging. In this regard, a marked increase in XO/CAT ratio (associated with H_2O_2 accumulation) was observed in both total peritoneal leukocytes (Fig. 6A.2) and macrophages (Fig. 6B.2) of old mice ($P < 0.001$) in comparison to those in adults. Moreover, this increased XO/CAT was also observed in macrophages from mature mice ($P < 0.05$). In contrast, long-lived mice showed in their total leukocytes and macrophages a lower XO/CAT than those observed in old ($P < 0.001$) and mature ($P < 0.01$ and $P < 0.05$, respectively) mice (Fig. 6A.2 and Fig. 6B.2).

3.5. Lipofuscin accumulation in macrophages and lymphocytes

Since lipofuscin is considered one of the best markers of aging at cellular level and its degree of accumulation is known to be also related

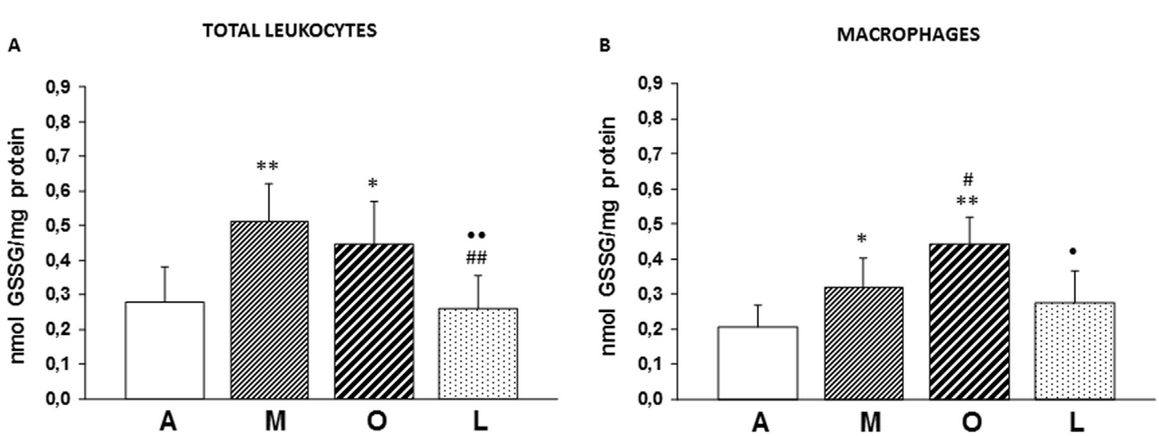

Fig. 4. Intracellular oxidized glutathione content (nmol GSSG/mg protein) in both total peritoneal leukocytes (A) and isolated peritoneal macrophages (B) from adult, mature, old and long-lived ICR-CD1 female mice. Data represent the mean ± SD of 6–10 values and each value being the mean of duplicate assays. *$P < 0.05$ and **$P < 0.01$ with respect to the value in adult mice. # $P < 0.05$ and ## $P < 0.01$ with respect to the value in mature mice. •$P < 0.05$ and •• $P < 0.01$ with respect to the value in old mice.

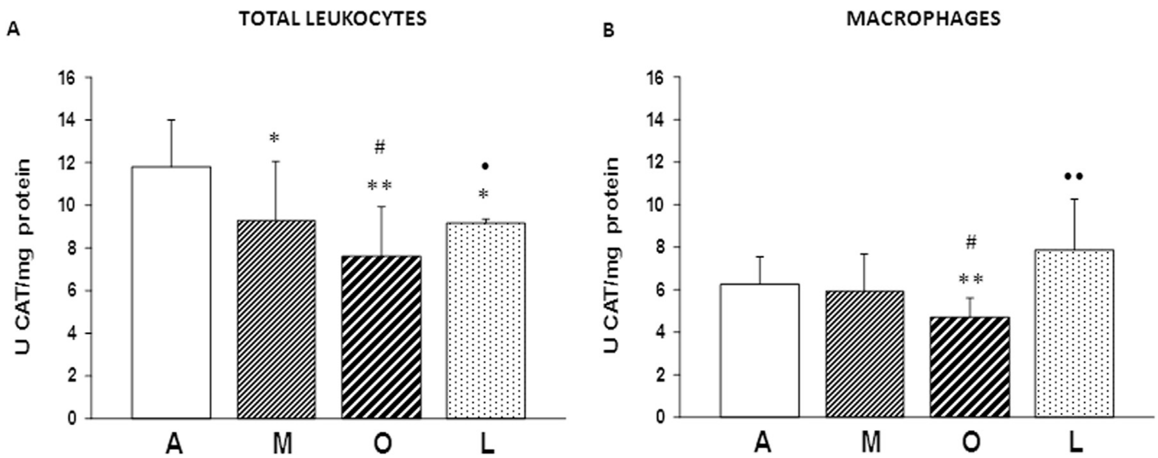

Fig. 5. Catalase activity (U CAT/mg protein) in both total peritoneal leukocytes (A) and isolated peritoneal macrophages (B) from adult, mature, old and long-lived ICR-CD1 female mice. Data represent the mean ± SD of 6–10 values and each value being the mean of duplicate assays. *$P < 0.05$* and **$P < 0.01$* with respect to the value in adult mice. # $P < 0.05$ with respect to the value in mature mice. •$P < 0.05$ and •• $P < 0.01$ with respect to the value in old mice.

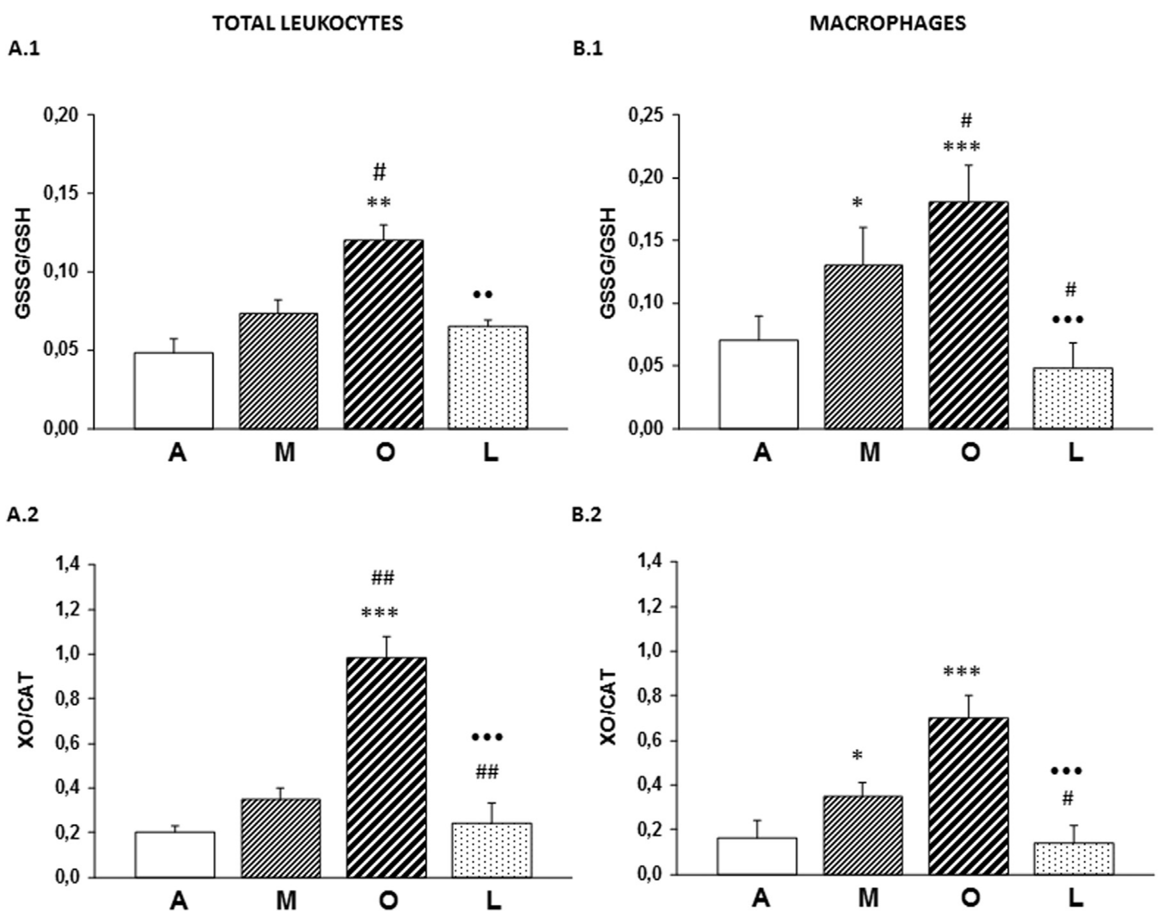

Fig. 6. Redox balance coefficient between both (1) the oxidized and reduced glutathione (GSSG/GSH) and (2) the activities of the pro-oxidant enzyme xanthine oxidase and the antioxidant catalase enzyme (XO/CAT) in both total peritoneal leukocytes (A) and isolated peritoneal macrophages (B) from adult, mature, old and long-lived ICR-CD1 female mice. Data represent the mean ± SD of 6–10 values and each value being the mean of duplicate assays. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ with respect to the value in adult mice. # $P < 0.0$ and ## $P < 0.01$ with respect to the value in mature mice. • •$P < 0.01$ and ••• $P < 0.001$ with respect to the value in old mice.

to oxidative stress and damage [17], the age-related changes in the intracellular accumulation of lipofuscin were measured in both peritoneal macrophages and T lymphocytes. The quantitative analysis of the autofluorescent lipofuscin load, inspected by fluorescence microscopy, is shown in Fig. 7 and Fig. 8. Lipofuscin accumulation significantly increased from adult to aged mice in peritoneal macrophages and T lymphocytes. Thus, in both cell population, the levels of lipofuscin observed in old mice were significant higher than those in adult ($P < 0.001$) and mature ($P < 0.01$ in lymphocytes; $P < 0.001$ in macrophages) mice. In contrast, lipofuscin levels in long-lived mice were significantly lower ($P < 0.05$ in lymphocytes; $P < 0.001$ in macrophages) than old mice, and in the case of macrophages, were similar to those observed in adult mice. Moreover, it is important to note that, in all ages analyzed, macrophages showed significantly higher accumulation of lipofuscin ($P < 0.001$) than lymphocytes. Thus, lipofuscin levels in macrophages from adult, mature, old and long-lived mice increased by

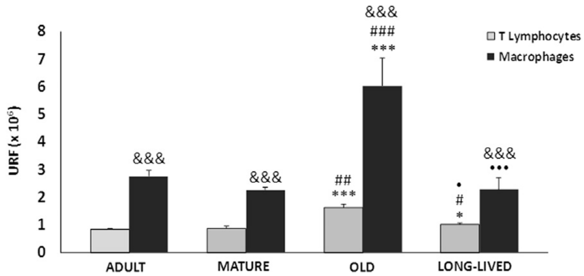

Fig. 7. Lipofuscin accumulation (URF) in both peritoneal macrophages and T lymphocytes from adult, mature, old and long-lived ICR-CD1 female mice. Data represent the mean ± SD of the values of 20 lymphocytes and 10 macrophages from each animal, and 3–5 animals were used from each group of age. *$P < 0.05$ and ***$P < 0.001$ with respect to the value in adult mice. # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ with respect to the value in mature mice. •$P < 0.05$ and ••• $P < 0.001$ with respect to the value in old mice. & & & $P < 0.001$ with respect to the value in T lymphocytes.

232%, 160%, 269% and 121%, respectively, in relation to lymphocytes.

The mean number, total area (pixel^2) and relative area fractions (%) of the lipofuscin granules, present in both isolated peritoneal macrophages and lymphocytes from mature, old and long-lived mice is shown in Fig. 9. Interestingly, in both T lymphocytes and macrophages, old mice showed a significant increase in either the mean number of lipofuscin granules ($P < 0.01$) (Fig. 9A), the total area of lipofuscin granules ($P < 0.01$ in lymphocytes; $P < 0.05$ in macrophages) (Fig. 9B) and the relative % of granules area fractions ($P < 0.001$) (Fig. 9C) compared to mature mice. In contrast, long-lived mice showed significantly lower number of lipofuscin granules in both types of cells ($P < 0.01$) (Fig. 9A), as well as a marked decrease in both total ($P < 0.01$) and relative ($P < 0.001$) area fractions of lipofuscin granules in relation to those observed in old mice (Fig. 9B-C). Finally, in relation to the type of cell analyzed, macrophages from old mice showed a higher mean number of lipofuscin granules density ($P < 0.05$) than those observed in lymphocytes (Fig. 9A). Moreover, macrophages from mature mice presented an increased total area of lipofuscin granules

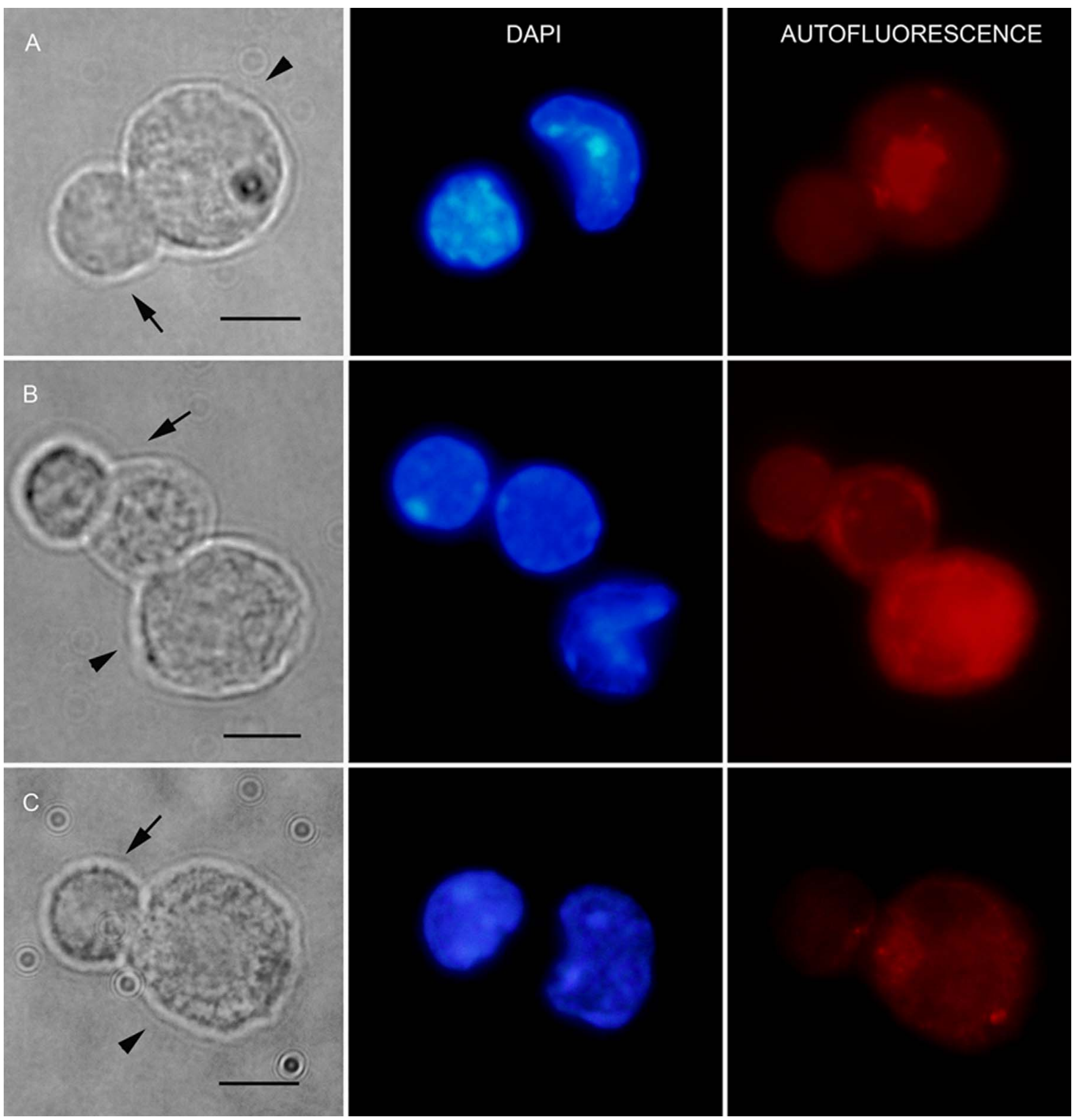

Fig. 8. Fluorescence microscopy images corresponding to the accumulation of lipofuscin in peritoneal macrophages (Δ) and T lymphocytes (→) from adult (A), old (B) and long-live (C) ICR-CD1 female mice, where the characteristic autofluorescence of lipofuscin (red) is observed. Cell nuclei were stained with 4′,6-diamino-2-phenylindole, DAPI (blue). The bar (-) corresponds to 5 µm.

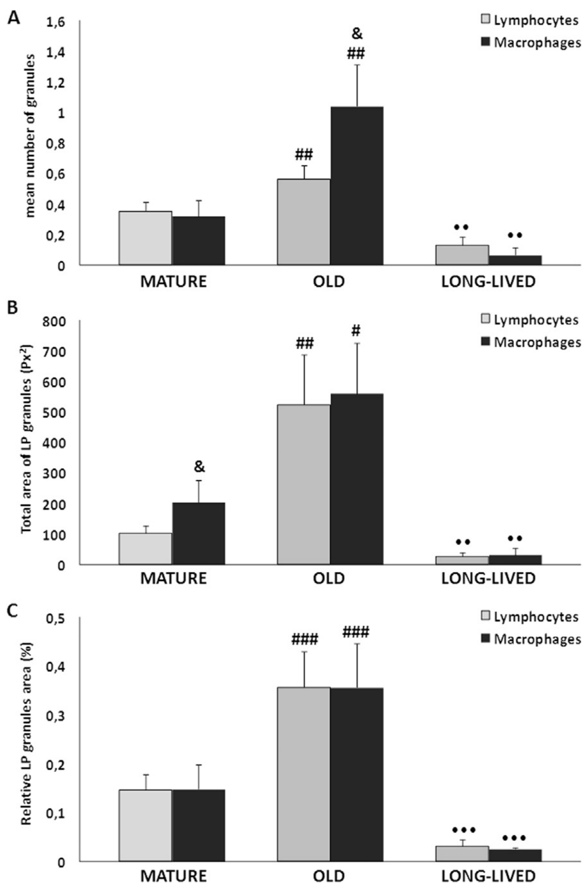

Fig. 9. Parameters of autofluorescent lipofuscin (LP) granules in both peritoneal macrophages and T lymphocytes from mature, old and long-lived ICR-CD1 female mice. (A) mean number of lipofuscin granules, (B) total area of lipofuscin granules (pixel^2) and (C) relative area fraction of lipofuscin granules (%). Data represent the mean ± SD of the values of 20 lymphocytes and 10 macrophages from each animal, and 3–5 animals were used from each group of age. # *P < 0.05, ## P < 0.01* and ### *P < 0.001* with respect to the value in mature mice. ••*P < 0.01 and* ••• *P < 0.001* with respect to the value in old mice. & *P < 0.05* with respect to T lymphocytes.

(*P < 0.05*) in relation to lymphocytes (Fig. 9B).

3.6. Age-related changes in macrophage functions

The maintenance of a good redox state and a low accumulation of cellular damage are of great importance for preserving immune function and achieving extreme old age [12]. In this regard, an imbalance between oxidants and antioxidant compounds in leukocytes from both aged animals and human contributes to the age-related impairment of their immune functions [1–3]. In addition, it has been described that severe lipofuscin accumulation in cells appears to result in a greatly decreased of cells functions, such as the phagocytosis, a key function of phagocytes [26]. Therefore, we hypothesized that the marked increase of both oxidative stress and lipofuscin accumulation observed in macrophages of old mice may contributes to the impairment of the macrophages functions in aged mice. To test this possibility, the age-related changes in both phagocytosis (ingestion of latex beads) and digestive capacity (intracellular $O_2^{\bullet-}$ production in response to the stimulation with PMA, a powerful activator of NADPH oxidase responsible of oxidative burst) were analyzed in peritoneal leukocytes. In this case, the macrophage functions were evaluated using the total population of leukocytes in order to better reproduce the *in vivo* immune response, and considering the observation that these immune responses may vary or be lost in purified isolates [27].

As shown in Table 1, the phagocytic capacity of macrophages was significantly impaired in old animals (*P < 0.05*) when compared with

Table 1
Phagocytic capacity (ingested latex beads) and digestive capacity (intracellular superoxide anion production in PMA-stimulated conditions) in total peritoneal leukocytes from adult, old and long-lived ICR-CD1 female mice.

	Adult (n=10)	**Old** (n=8)	**Long-lived** (n=6)
A) Phagocytic capacity			
Phagocytic Index	322 ± 49	260 ± 11 *	362 ± 29 ••
B) Superoxide anion production in response to PMA			
nmol $O_2^{\bullet-}$/10 ^{6} cells	15.78 ± 5.91	11.69 ± 2.16 *	18.30 ± 1.47 ••
% stimulation	203 ± 5	89 ± 5 ***	213 ± 8 •••

Data represent the mean ± SD of the values from the number of animals used at each experimental time (in parentheses) and each value being the mean of duplicate assays. **P < 0.05* and ****P < 0.001* with respect to the value in adult mice. ••*P < 0.01 and* ••• *P < 0.001* with respect to the value old mice.

adults. The digestive capacity was also decreased in old animals (*P < 0.05*) as compared to adults. In contrast, the peritoneal macrophages from extreme long-lived animals showed intact the phagocytic functions studied, the values being similar to those observed in adults. Thus, peritoneal macrophages from long-lived mice showed increased phagocytosis and digestive capacity (*P < 0.01*) compared with macrophages from old mice (Table 1). Interestingly, having established the basal $O_2^{\bullet-}$ production status of peritoneal leukocytes from old mice (being their values 100%), the PMA-stimulation resulted in a marked decreased in the percentage of $O_2^{\bullet-}$ production (89% stimulation; *P < 0.001*) compared to that observed at baseline in old animals. In contrast, adult and long-lived mice showed in their leukocytes a higher PMA-stimulated $O_2^{\bullet-}$ production in relation to their basal levels (Table 1).

Taken together, these data confirm an alteration of the macrophages functions with the age and reveal that those animals surviving to extreme old age, in fact, display an immune response similar to that seen in adults.

3.7. Age-related changes in peritoneal leukocytes population

The age-related changes in the immune cells involve impairment of functions but also affect the composition, quality and rate at which the different immune cells populations are produce. In this regard, several of changes observed in the immune response have been attributed to the modifications of immune cells subpopulations with aging [2]. Therefore, due to the age-related decline observed in the functions of macrophages (decreased phagocytosis and digestive capacity in old mice), we next assessed the age-related changes in peritoneal leukocytes population, measuring by flow cytometry the expression of the cells surface markers CD11b, CD19 and CD3, markers of macrophages, B and T lymphocytes differentiation, respectively.

Age-related remarkable changes were found with respect to the percentages of positively labeled leukocytes for CD11b, CD19 and CD3 (Table 2). Thus, mature and old mice showed a significant increase in the percentage of macrophages (*P < 0.05* and *P < 0.001*, respectively) compared with adults. In contrast, a lower percentage of both B and T cells were observed in the peritoneal suspensions of mature (*P < 0.05* and *P < 0.001*, respectively) and old (*P < 0.05* and *P < 0.01*, respectively) mice with respect to adult mice. In relation to the long-lived animals, decreased percentages of macrophages and B lymphocytes (*P < 0.05*) were observed in relation to the adult group, whereas no statistically significant differences were observed in the percentages of T lymphocytes. Furthermore, in relation to mature and old mice, long-lived mice showed a significant decrease (*P < 0.001*) and increase (*P < 0.01*) in the percentage of both macrophages and T cells, respectively.

Table 2
Percentages of membrane expression of the leukocytes differentiation antigens CD11b (macrophages), CD19 (B lymphocytes) and CD3 (T lymphocytes) of peritoneal leukocytes from adult, mature, old and long-lived ICR-CD1 female mice.

	Adult (n=6)	**Mature** (n=6)	**Old** (n=5)	**Long-lived** (n=4)
Macrophages (% CD11b+)	6.65 ± 0.84	11.62 ± 2.65*	14.48 ± 0.23***#	4.46 ± 0.89*###•••
B lymphocytes (% CD19+)	57.19 ± 13.63	54.54 ± 8.93*	49.71 ± 19.59*	49.73 ± 12.10**##
T lymphocytes (% CD3+)	28.87 ± 5.40	10.59 ± 4.11***	14.69 ± 8.17**	26.46 ± 7.13##••

Data represent the mean ± SD of the values from the number of animals used at each experimental time (in parentheses) and each value being the mean of duplicate assays. **P < 0.05*, ***P < 0.01* and ****P < 0.001* with respect to the value in adult mice. *# P < 0.05, ## P < 0.01* and *### P < 0.001* with respect to the value in mature mice. *••P < 0.01 and ••• P < 0.001* with respect to the value old mice.

4. Discussion

This is the first study to analyze the age-related changes in several oxidative stress and function parameters, as well as in the lipofuscin accumulation in peritoneal macrophages from mice, including a group of extreme long-lived mice, which represent the best living example of successful aging. Since it has been proposed that phagocytic cells may be the immune cells that most contribute to the oxidative stress and damage associated with immunosenescence [1,2], our study was performed in isolated peritoneal macrophages, as well as in the total population of peritoneal leukocytes, containing mainly B and T lymphocytes, macrophages, and NK cells, in order to evaluate the possible age-related differences in the parameters evaluated depending on the type and cellular composition. However, due to the limitations in obtaining isolated peritoneal lymphocytes, and the low number of lymphocytes recollected, the age-related differences between lymphocytes and macrophages could only be analyzed in several parameters, such as the activity of the pro-oxidant enzyme XO and in the lipofuscin accumulation.

Macrophages are the central effector cells of the innate immune system, as well as play a relevant role in the maintenance of tissue homeostasis, responding to the physiological changes and the challenges from outside [28,29]. However, these homeostatic functions can be subverted by chronic insults, resulting in a causal association of macrophages with age-related diseases [28]. In this regard, macrophages release a vast range of inflammatory mediators, including cytokines (e.g. TNF-α, IL-6 and IL-1β) and chemokines that are central to initiation and propagation of the inflammatory process [29]. However, although this inflammatory adaptive macrophage response is initially beneficial because they facilitate the clearance of invading organisms, becomes maladaptive when is not quickly controlled, leading to a state of chronic low-grade inflammation [28,30]. In these circumstances, macrophages have a harmful rather than repairing effect and they cause lesions. For example, in the context of obesity, cytokines release from resident macrophages in adipose tissue, resulting in a proinflammatory microenvironment, which has been proposed to be the major contributors to the chronic low-grade inflammation seen in aging [31]. Moreover, since oxidative stress and inflammation process are able to induce and exacerbate one another (both indirectly and directly), the low-grade of inflammation caused by macrophages also triggers substantial collateral tissue oxidative damage, due to the toxic actions of the oxidant compounds produced by themselves, which contributes to disease progression [28]. In addition, age-related impairments in macrophage function are likely to have important consequences for the health of older population. It has been reported that aging in macrophages impacts on many processes including toll-like receptor (TLR) signaling, polarization, phagocytosis, and wound repair [29]. Therefore, a detailed understanding of the impact of aging on macrophages is required in order to develop therapeutics that will boost immune responses in the older population. In this regard, it is important to assess the causative link between age-related alterations in the function of macrophages and the onset and progression of chronic age-related increased of oxidative stress and damage, which is the base of aging.

The results of this study demonstrated that peritoneal leukocytes from old mice, and especially macrophages, had an increased oxidative stress, high accumulation of lipofuscin, and impaired immune functions in relation to adults. This alteration in the oxidant balance, which is, at least in part, mediated by an increased in both XO activity and expression, increased levels of $O_2^{\cdot-}$ and ROS along with high GSSG content, as well as by decreased CAT activity and lower levels of GSH, could contribute to the higher accumulation of lipofuscin, as well as to the impairment of both phagocytosis and digestive capacity observed in the aged macrophages. Moreover, it is important to note that macrophages of old mice showed higher XO activity and accumulation of lipofuscin than the lymphocytes, suggesting that phagocytic cells could contribute in a relevant manner to the oxidative stress and damage associated with immunosenescence. In contrast, long-lived mice showed in their peritoneal leukocytes, and mainly in macrophages, a well-preserved redox state, in terms of XO activity and expression, oxidized compounds production, proper function of catalase antioxidant enzyme and low levels of lipofuscin in its macrophages. This preservation of their redox state and the lower accumulation of cellular damage, which resembles that of adult mice, could contribute to the maintenance of their immune functions and consequently of health and longevity.

The XO is an evolutionarily conserved enzyme involved in multiple features of innate immunity, which play an important role in the fight against pathogens, due to its direct and/or indirect contribution to the formation of ROS and RNS [5,6]. However, when the activity of this enzyme is not well regulated, as observed in mature and old mice, where an exacerbated increased of its activity and/or expression has been found in both total peritoneal leukocytes and macrophages, this may lead to excessive ROS formation, increasing oxidative stress. Interestingly, in isolated lymphocytes, XO activity did not vary at the different ages studied, suggesting that the increased XO activity observed in the total population of leukocytes is mainly due to the observed increase XO activity in peritoneal macrophages. The increased XO activity/expression observed in the total population of peritoneal leukocytes from old mice confirms previous findings of our groups [12,20], and is in accordance to what takes place in other tissues along the aging process [20,32,33]. Although, to our knowledge, there are no studies regarding the age-related changes in the activity of XO in phagocytes, a recent work has demonstrated that the ROS-generates by XO mediate NLRP3-inflammasome activation in macrophages, triggering the release of pro-inflammatory cytokines (i.e. IL-1β) [34], whereas its inhibition (e.g. allopurinol) attenuates the activation of NLRP3, decreasing the release of these cytokines [34–36]. Although the mechanisms by which XO is linked to the inflammation process are not entirely clear yet, some authors also propose that

XO is also be involved in the activation of TLR in phagocytes, since its inhibition results in a decreased TLR-2-mediated IL-1β secretion [37,38]. Therefore, these pathways and the increased XO activity and expression in peritoneal leukocytes, and especially macrophages of old mice, could contribute to the increased of oxidative-inflammatory stress and cellular oxidative damage, especially in macrophages.

The increased XO activity in total leukocytes and macrophages of old mice was coincident with the enhanced content of levels of both intracellular $O_2^{\cdot-}$ and ROS (mainly, H_2O_2), whereas extremely long-lived animals showed well-preserved levels of oxidizing compounds, being similar to those observed in adults. Similar results have been described in other studies assessed in total peritoneal leukocytes of old mice [12,39], whereas a decrease in basal $O_2^{\cdot-}$ levels of granulocytes has been reported in centenarians [40]. Although the increased ROS levels could be indicative that the leukocytes, and especially macrophages, would be performing a good defensive response [1,2], the fact that the old mice present exacerbated intracellular basal levels of $O_2^{\cdot-}$, ROS, and GSSG, together with alterations in their antioxidant systems, as will be described later, could lead to an oxidant-antioxidant imbalance in these cells, contributing to the increase of oxidative stress and damage. Furthermore, it is important to note that in old mice, differences in $O_2^{\cdot-}$ and ROS levels were found depending on the type of immune cells analyzed. Thus, peritoneal macrophages showed higher basal levels of $O_2^{\cdot-}$ than those observed in the total leukocytes population, whereas the ROS production where higher in total leukocytes than in macrophages. This suggests that the presence of lymphocytes influences the degree of activation of macrophages. In fact, it is known that T lymphocytes and macrophages are not only capable of activating each other but they are also able to induce immune suppressive effects [41]. Thus, the age-related changes in $O_2^{\cdot-}$ and ROS production in macrophages, at least in part, is also related to the type of the T cell repertoire that they are accompanied, and which also is deep remodeling along aging [27].

In regard to the antioxidant defenses, old mice showed a decrease in catalase activity and GSH levels in their peritoneal leukocytes, and especially in their peritoneal macrophages. In contrast, peritoneal macrophages from long-lived animals showed a well-preserved catalase activity and GSH levels, being similar to adults, whereas in the total peritoneal leukocytes, both antioxidant parameters decreased in comparison to adults. In this context, some studies have found a relationship between catalase activity and longevity [40], which could be related to the important role this enzyme plays in tolerance to oxidative stress and adaptive cell response (because it acts only in the presence of high H_2O_2 levels). Thus, the catalase activity is a key mechanism in preventing endogenous damage caused by oxidative burst and chronic inflammation, leading to long-term preserved immune cell function and longevity [12]. The present results are in consonance with previous studies from our group, which have shown decreased catalase activity and low levels of GSH in leukocytes from both old and prematurely aged mice [12,39]. This further supports the impairment of these parameters as an early driver of aging of the immune system [2,12]. Moreover, the peritoneal leukocytes from long-lived mice and peripheral blood phagocytes from human centenarians (mainly neutrophils) have shown increased or similar catalase activity and GSH levels as compared with adult and young, which contributes to the maintenance of low oxidative stress in these immune cells [12–14]. Although, to our knowledge, there are no studies about the age-related changes in the catalase activity of isolated murine peritoneal macrophages, it is known that endogenous catalase plays an important role in the polarization of tissue macrophages, inhibiting inflammation and reducing nitric oxide production in response to LPS [42,43]. Moreover, the inhibition of this enzyme increases ROS production, promoting inflammatory response in alveolar macrophages [44]. This suggests that the low catalase in aged peritoneal macrophages, could contributes to an activation of inflammation in these cells. In addition, the decrease in GSH levels in both total peritoneal leukocytes and macrophages from old animals compared with adults were coincident with both enhanced intracellular GSSG content and GSSG/GSH ratio. Under normal physiological conditions GSH is significantly favored over GSSG, however, pathological conditions causing oxidative stress results in increased GSSG levels and GSSG/GSH ratio [23]. Thus, although macrophages and total leukocytes showed lower levels of GSSG than GSH in all ages studied, it is important to note that older mice showed a marked increase in GSSG in both cell populations. By contrast, successfully aged animals showed in their peritoneal macrophages and total leukocytes GSSG levels and GSSG/GSH ratio similar to adult individuals. These results confirm previous results of our group assessed in total peritoneal leukocytes from old and long-lived mice [12]. In this context, it is important to note the crucial role that the cycle of glutathione plays protecting against oxidative stress especially in phagocytes. In fact, there is a remarkable drop in the cellular content of GSH within minutes after activation and a decreased bactericidal capacity of phagocytes in individuals deficient in this particular redox system [45]. Although, to our knowledge there are no studies which have been analyzed the age-related changes in the GSH homeostasis in murine isolated peritoneal macrophages, other research assessed in alveolar macrophages, showed that when the GSH availability in macrophages is limited, cellular functions such as phagocytosis and respiratory burst become compromised [46].

An efficient removal of ROS can only be successful when there is a good balance between the different phases or stages of the diverse antioxidant routes. Thus, maintaining adequate levels of antioxidants is essential to prevent or even manage changes in redox balance during disease development [47]. In this sense, due to the increased XO activity and the decreased catalase activity observed in aged macrophages we decided to analyze the different modulation of the pathway XO and catalase activities throughout aging. In our study, a higher XO/CAT ratio was observed in both total peritoneal leukocytes and macrophages, suggesting a lower efficiency in catalase response, and therefore, a greater accumulation of H_2O_2 occurs in these cells compared to adults. Since H_2O_2 is more stable than other ROS, and can diffuse through membranes, it can cause oxidative damage at long distances from its origin [48]. In this context, it has been demonstrated that aged murine macrophages suffer high susceptibility to oxidative stress, due to that these cells decreased their cell growth and DNA repair capacity when treated with H_2O_2 [49]. Furthermore, the increased XO/CAT ratio was slightly higher in total peritoneal leukocytes than in macrophages, suggesting that lymphocytes would also contribute this increase when are present in the total population of leukocytes; this is probably due to their decreased catalase activity, even no changes were observed in the XO activity of aged lymphocytes. Interestingly, long-lived mice presented an optimal XO/CAT balance, which could be explained as a compensatory mechanism to deal with ROS levels. Thus, taken together, our results demonstrated that long-lived animals show in their immune cells a well-preserved redox state, in terms of antioxidant defenses and oxidant compounds, which could be related to the well-conserved function in response to stimuli of these cells. Surprisingly, long-lived animals showed marked differences in GSH content in relation to adults depending on the cell type analyzed in the peritoneum. This could be explained by the capacity of macrophages to regulate the intracellular GSH levels in lymphocytes [50]. Thus, the fact that long-lived animals showed lower GSH content than adult in the total population of peritoneal leukocytes, whereas isolated macrophages preserved their GSH content, could be due to macrophages strongly augment the amount of thiol released when DNA synthesis of cycling T cell clones occurs [50]. This suggests that when both cell populations are together, the lymphocytes may be used the GSH released by macrophages in order to preserve their proliferative capacity, as previously have been reported in peritoneal leukocytes from long-lived mice [12].

During the aging process, immune cells, and mainly phagocytes, produce the highest levels of oxidant and pro-inflammatory compounds, thereby being especially sensitive to the age-related oxidative cellular damage [1,2]. Even so, all these cells have self-repairing mechanism that turn over and reuse their damaged macromolecular constituents. However, during aging, the capacity to repair oxidative-stress-induced cellular damage is decreased, and not all damaged structures are being removed, contributing to a gradually accumulation of damaged compounds in the cell [51]. This is the case of lipofuscin, which is one of the most important manifestations of ROS-induced damage that occurs within the lysosomal compartment of most aging eukaryotic cells [17,51]. It is important to note that its degree of accumulation is known to be also related to oxidative stress and damage, as well as with the impairment of essential cell functions, contributing to many age-related diseases [17]. Our study demonstrates that murine peritoneal lymphocytes and macrophages, in resting state, accumulate lipofuscin in an age-dependent manner, showing older mice the highest levels of lipofuscin, whereas long-lived mice showed preserved levels of this “age pigment”. Moreover, it is important to note that this age-related lipofuscin accumulation compromises the correct function of these cells in a direct or indirect manner. Our results are in agreement with others previously reported in the literature. For instance, Beregi et al. (1991) [52] examined “in vivo” some immunological variables from peripheral blood lymphocytes in centenarians and healthy subjects (60–89 years-old). The results showed the presence of lipofuscin in the cytoplasm of lymphocytes that significantly increased with age. Similarly, Gerland et al. (2004) [53] showed, in long-term cultured human CD8+ T-lymphocytes, a significant progressive accumulation of lipofuscin with aging together with a progressive intolerance to activation.

Regarding macrophages, recent studies reported that macrophages are also involved in the process of lipofuscin accumulation [54–56]. For example, Luhmannet et al. (2009) [55] showed that the accumulation of macrophages with lipofuscin granules in the subretinal space is a normal age-related process that is accelerated in Ccl2−/− mice that show phenotypic features similar to age-related macular degeneration, while Nag (2015) [56] reported macrophages with abundant irregular lipofuscin loaded-melanosomes of variable size in the aging human choroid, suggesting that damaged melanocytes are cleared by these phagocytes. It is important to highlight that, in all the ages analyzed in our study, macrophages showed significantly higher accumulation of lipofuscin than lymphocytes, which could be explain by the fact that macrophages produced higher levels of ROS than lymphocytes, due to their role in host defense and their high glycolytic metabolism (macrophages display more mitochondrial activity than lymphocytes) [57]. The higher accumulation of lipofuscin in aged macrophages, could bring negative effects on the important turnover functions performed, such as loss of autophagy and/or a decline in phagocytosis [26,51]. Thus, a lipofuscin-loaded cell would not continue phagocytosis until its vacuolar apparatus is cleared of undigested material [26,51], whereas the loss of autophagy incapacitates the cell to completely remove damage cellular structures in the progressive accumulation of garbage (e.g. cytosolic protein aggregates) [26,51]. Moreover, it has been proposed that the accumulation of lipofuscin inside the lysosomal compartment results in prolongation of mitochondrial life span with accumulation of enlarged functionally effete mitochondria, and ensuing decline in ATP production, increased formation of ROS, accelerated formation of lipofuscin and, finally, lysosomal labilization with activation of apoptotic or necrotic pathways [17]. All these alterations at the cellular level inevitably lead to progressive functional decline, decreased adaptability, and an increased probability of disease and death for the organism. Our results are in agreement with other study in which it has been described that the degree of lipofuscin accumulation in retinal epithelial cells is directly related with the decline of the phagocytotic capacity and the ability to eliminate ROS of these cells [26]. Consequently, many biomolecules become oxidized and undegradable, promoting oxidative stress dramatically, which could also enhance lipofuscin formation, contributing to a vicious circle of oxidation and cellular injury damage. Furthermore, since it has been discovered that lipofuscin may act as a danger signal, which in turn lead to the secretion of inflammatory cytokines and chemokines by macrophages and other cells, through activation of the NLRP3-inflammasome or TLRs receptors [18], our results further support the link between oxidation and inflammation in macrophages. In turn, the higher lipofuscin accumulation that old mice show in their macrophages, could stimulate TLRs receptors and NLRP3-inflammasome, leading to the release of proinflammatory cytokines, as well as to other signaling pathways mediators, such as nuclear factor NF-KB or NADPH-oxidase complex [4,58], which increase ROS production, and therefore contributing in a relevant manner to a higher oxidative-inflammatory state and, consequently, to increase the rate of aging. Finally, since an enhancement lipofuscin formation by oxidative stress could be decrease by administration of antioxidants [17], the lower levels of lipofuscin observed in macrophages from adult and long-lived mice could be explained by the fact that these individuals also showed a well conserved redox balance, with efficient antioxidant defenses and lower production of oxidizing compounds, which lead to lower levels of oxidative damage and then to smaller lipofuscin levels. This remarkable finding suggests a good maintenance of the self-repairing mechanism.

The changes that occur with age in the function of the immune cells could be closely linked, at least in great part, to the age-related chronic oxidative stress to which they are exposed during the course of time, as well as with the accumulation of oxidative damage in their biomolecules [1,2]. In this context, the impaired response observed in old mice (decreased phagocytosis and digestive capacity) could be mediated by the observed increase of both oxidative stress and lipofuscin accumulation affecting these immune cells. This indicate an alteration in the bactericidal activity of aged macrophages, which would not be able to activate in the presence of the stimulus, as indicated by the low digestive capacity observed in old mice. Because macrophages are an essential component of both innate and adaptive immunity, altered function of these phagocytic cells with aging may play a key role in immunosenescence. Our results confirm previous work described by our group and others authors, in which a decline in the phagocytic capacity and in the ability to generate ROS in the respiratory burst have also been reported to occur in phagocytes form both aged animals and elderly humans [12,13,59,60]. Moreover, altered phagocytosis could contribute to oxidative cellular damage, due to the fact that free radicals can be released to the extracellular space with concomitant damage to the structure of phagocytes and neighboring tissues [1]. By contrast, long-lived mice preserved their macrophage functions, which could be explain by the well-maintenance of the redox balance and the lower lipofuscin accumulation observed in their macrophages. In this regard, several studies in both humans and mice has been found that a decreased phagocyte function is related to shorter life span, suggesting that preservation of this function, as occurs in peritoneal macrophages from long-lived mice, could also contribute to extend survival [12,14]. It is important to note that, the amount of ROS produced in stimulated phagocytes is significantly higher than in the other cell types. Therefore, general and phagocyte-specific mechanisms have been co-evolved with the radical generating machinery of phagocytes [45]. Since the outcome of local inflammation can directly depend on the antioxidant capacity, the optimal antioxidant balance observed in macrophages from long-lived mice may contributes to an adequate elimination of pathogen, whereas the imbalance oxidant-antioxidant status found in aged macrophages contributes with minimal acute tissue damage to progression towards a systemic inflammatory response [28,31,45].

The effectiveness of the immune responses and the balance of the redox status could be influenced by immune cell population changes (in terms of frequency or counts) [1]. In our study, the flow cytometry analyses carried out in the total populations of peritoneal leukocytes showed an increased in percentage of macrophages (CD11b+), as well as a decreased percentage of both B (CD19+) and T (CD3+) lymphocytes, in the peritoneum of mature and old mice in relation to adults. The increased number of macrophages observed in old mice could be explained as a compensatory mechanism towards the loss of functions observed in these cells. However, an increase in the number of a specific immune cell type does not imply a better performance of these cells. In addition, the age-related increased in basal XO activity/expression and $O_2^{\cdot-}$ and ROS intracellular production observed in the peritoneal leukocytes, and their preservation in long-lived animals, could also be explained by the differences observed in the percentages of macrophages in each group of age.

5. Conclusion

In the last years cumulative evidence has indicated a tight cause-effect link between *oxi-inflamm-aging* and immunosenescence. In this context, it has been suggested that phagocytes, such as macrophages, which are present in all animals, are the immune system cells which are more implicated in the chronic oxidative and inflammatory stress of senescence, due to their higher production of oxidant and inflammatory compounds in relation to lymphocytes [1,2]. The results of the present study demonstrate that macrophages from old mice have a marked increase of oxidative stress and damage in relation to adults, confirmed by a higher production of oxidizing compounds (XO activity/expression and intracellular basal levels of $O_2^{\bullet-}$, ROS and GSSG), a decreased antioxidant defenses (catalase activity and GSH levels), as well as by a higher lipofuscin accumulation and XO/CAT and GSSG/GSH imbalance. This fact appears to be the basis of their age-related function deterioration (decreased phagocytosis and digestive capacity). In contrast, macrophages of long-lived mice maintain a well-controlled redox state, which seems to be, at least in part, mediated by preserved levels of XO activity/expression, low basal $O_2^{\bullet-}$, ROS production and GSSG levels, as well as preserved catalase activity and GSH levels, low lipofuscin accumulation and no altered macrophage functions. Therefore, since an appropriate immune function is related to better health and longevity, these data suggest the importance of maintaining both redox status and low lipofuscin accumulation during the aging process. Moreover, since lipofuscin is strongly associated with aging, and its formation is promoted by oxidative stress, the fact that, in all the ages studied, macrophages showed higher lipofuscin accumulation, together with higher XO activity, than lymphocytes, supports the idea that macrophages contribute more than lymphocytes to the chronic oxidative stress and oxidized-damaged accumulation associated with aging, especially in old ages. Since it has been discovered that both lipofuscin and ROS-generates by XO may act as danger signals, which in turn lead to the secretion of inflammatory cytokines, our results could supports the link between oxidation and inflammation in macrophages.

Finally, since the relationship between the redox state and the function of immune cells influence the rate of aging and lifespan, our results suggest that the determination of oxidative stress and immune functions parameters, together with the quantification of lipofuscin accumulation, in macrophages, can be useful markers of rate of aging and as predictor of longevity. In addition, these findings demonstrate that macrophages play a central role in “oxi-inflamm-aging”, these cells providing a useful model to elucidate the molecular mechanisms underlying the aging process. However, further studies are needed to identify the oxidative-inflammatory molecular mechanisms of phagocytes in aging, in order to find targets of strategies that allow the maintenance of a good health, preventing the age-related diseases and associated premature death, and consequently, promote a healthy longevity.

Conflict of interest

The authors declare no conflict of interest.

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